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Iron is an essential dietary element that supports oxygen transport, nutrient metabolism and protein synthesis; however, dysregulation of iron biology can be detrimental to health. The importance of iron homeostasis is particularly apparent in the brain where deficiencies result in impaired cognition and accumulations are associated with neurodegenerative diseases. Despite the observation that altered iron status is associated with impaired brain health, little is known about what drives iron dysregulation in the brain.

Obesity is associated with increased risks for altered systemic iron status and neurodegeneration, but the effects of obesity on iron biology in the brain are uncertain. The following study characterizes changes in brain iron biology resulting from high fat diet-induced obesity in a mouse model. The key findings from this study include the observation of attenuated iron concentration changes between late development and adulthood, as well as increased alpha-synuclein expression, and evidence of lipid peroxidation in certain iron-rich brain regions of the obese mice compared to controls. These findings demonstrate that obesity is sufficient to alter brain iron biology and that the alterations occur in a regionally differentiated manner. Further, the observation of elevated alpha-synuclein and lipid peroxidation in regions where iron biology is also altered indicates that the changes brought on by obesity may be involved in iron-related neurodegenerative processes.

THE EFFECTS OF DIET-INDUCED OBESITY ON REGIONAL BRAIN IRON
BIOLOGY

by

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TABLE OF CONTENTS

| | Page |
|---|------|
| LIST OF TABLES | vi |
| LIST OF FIGURES | vii |
| CHAPTER | |
| I. INTRODUCTION | 1 |
| II. REVIEW OF THE LITERATURE | 5 |
| Introduction..... | 5 |
| Brain Iron Transport | 6 |
| The Brain and Iron Status | 8 |
| Obesity and Iron Status..... | 9 |
| Obesity and Neurodegeneration..... | 13 |
| Alzheimer's Disease | 13 |
| Parkinson's Disease | 15 |
| Alpha-Synuclein | 17 |
| Conclusion | 18 |
| III. THE EFFECTS OF DIET-INDUCED OBESITY ON REGIONAL BRAIN IRON BIOLOGY..... | 20 |
| Abstract..... | 20 |
| Introduction..... | 21 |
| Methods..... | 25 |
| Animals | 25 |
| Diet..... | 26 |
| Hematocrit and Plasma | 26 |
| Tissue Collection | 27 |
| Iron Analysis..... | 27 |
| RNA Isolation and cDNA Synthesis..... | 28 |
| Real-Time Polymerase Chain Reaction (RT-PCR) | 29 |
| F ₂ -Isoprostane Assay | 29 |
| Data Analysis | 29 |
| Results..... | 30 |
| Descriptive Data..... | 30 |
| Systemic Iron Concentration..... | 31 |
| Regional Brain Iron..... | 32 |

| | |
|---|----|
| mRNA Expression of Iron Homeostasis Proteins..... | 33 |
| F ₂ -Isoprostane Formation..... | 35 |
| Discussion..... | 36 |
| Obesity and Midbrain Iron..... | 41 |
| Obesity and Thalamus Iron..... | 42 |
| Alpha-Synuclein and Brain Iron..... | 42 |
| Implications and Future Study..... | 43 |
| IV. EPILOGUE..... | 46 |
| REFERENCES..... | 52 |

LIST OF TABLES

| | Page |
|---|------|
| Table 2.1. High Fat Diet and the Risk of Parkinson's Disease..... | 16 |
| Table 3.1. Total Weight and Adipose Weight by Diet. | 31 |
| Table 3.2. Whole Blood Hematocrit and Plasma Iron Concentration. | 31 |
| Table 3.3. Selected Systemic Tissue Iron Concentration ($\mu\text{g}/\text{mg}$ protein) | 32 |

LIST OF FIGURES

| | Page |
|---|------|
| Figure 1.1. Working Model. | 2 |
| Figure 1.2. Study Design | 4 |
| Figure 3.1. Study Design | 25 |
| Figure 3.2. Select Regional Iron Concentrations | 33 |
| Figure 3.3. Brain Iron Metabolism Related mRNA Expression | 34 |
| Figure 3.4. Liver Iron Metabolism Related mRNA Expression | 35 |
| Figure 3.5. F ₂ -Isoprostane Assessment | 36 |
| Figure 3.6. Results Summary | 37 |

CHAPTER I

INTRODUCTION

Apart from the known causes of altered iron status (e.g. insufficient dietary iron, impaired absorption of dietary iron, blood loss, and genetic abnormalities) a link to obesity has also been established [1–5]. The cause of the systemic iron deficiency seen in obesity has more recently been associated with low-grade chronic inflammation and increased serum concentrations of the iron regulatory protein hepcidin [2–4,6]. However, a full understanding of the iron regulatory changes brought on by obesity and, in particular, those that affect the brain have yet to be characterized and warrant further study.

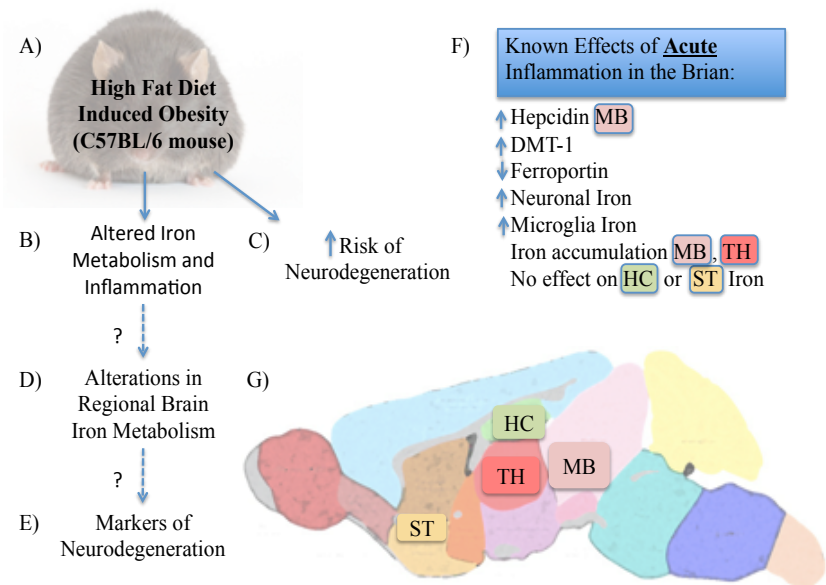
Research has demonstrated that certain regions of the normal brain contain higher iron concentrations than the brain as a whole [7,8]. The iron-rich regions include the substantia nigra, striatum, hippocampus, and thalamus among others. Erikson et al. and Piñero et al. reported that these brain regions are differentially affected by neonatal iron deficiency and subsequent iron repletion [9,10]. The effects of iron deficiency during infancy on brain development and cognition have been studied and shown to cause neurological impairments that can last decades [11]. Excessive iron in the brain can also be detrimental. Neurodegenerative diseases have been associated with the accumulation of iron in specific brain regions; however, the relevance of these findings to the disease etiology is currently unclear [12]. Separately, obesity and high fat intake are associated

with two of the most prominent neurodegenerative diseases, Alzheimer's disease and Parkinson's disease [13–16]. Therefore, our study was conducted to observe the effects of high fat diet-induced obesity on brain iron biology to determine if there is a link between obesity-induced altered brain iron and neurodegenerative processes. The working model below characterizes the current knowledge and gaps to be addressed (Figure 1.1.).

Figure: 1.1.
Working Model.

Obesity is associated with an increased risk of iron deficiency and alterations in systemic iron biology likely due to chronic inflammation [1,2,6]. A) To study the effects of obesity on brain iron biology we used a mouse model (C57BL/6), which is known to develop obesity [17,18] with

characteristics similar to human physiological changes including: B) elevated inflammatory markers [2,6,19,20], alterations in iron metabolism proteins [20,21], and iron deficiency without anemia [1,22,23]. C) Obesity is also known to increase the risk of developing neurodegeneration [13,15,16] D) We hypothesized that the changes in systemic iron biology brought on by obesity extended to regional brain iron biology in adolescence and adult mice. E) Further, using assays to determine if alpha-synuclein gene expression and lipid peroxidation were increased, we sought to associate changes in regional brain iron biology with indications of neurodegenerative disease. F) Acute inflammation has been shown to alter brain iron biology in a manner that is differentiated across cell types and brain regions, lending further support to our hypothesis that obesity may alter iron biology in the brain in a regionally distinct manner [24–27]. G) Our study focused on four iron-rich brain regions [7,8] that are associated with Alzheimer's disease (Hippocampus, HC; Thalamus, TH) [28–32], and Parkinson's disease (Midbrain, MB; Striatum, ST) [7,33–38]. (Brain image courtesy of www.gensat.org)



We utilized a well-characterized murine model (C57BL/6J) of high fat diet-induced obesity to assess changes in brain iron biology [17,18]. The aims of our study were to:

1. Determine changes in brain iron concentration and the expression of genes related to iron biology in four iron-rich regions of the mouse brain including: the midbrain, the thalamus, the striatum, and the hippocampus, in mice experiencing altered systemic iron biology associated with obesity [7,8].
2. Evaluate iron-related changes in these brain regions across developmental stages: adolescent (6 week) and adult (20 week), within and across dietary groups and time points.
3. Compare alpha-synuclein gene expression and a marker of lipid peroxidation between the obese and control mice to determine if early signs of neurodegenerative disease are present in the brain regions of obese mice.

Based on previous un-published data, we hypothesized that obesity would alter regional iron accumulation with a significant increase in the midbrain and varying degrees of change in the remaining regions. Additionally, we hypothesized that the effects of obesity on brain iron biology would differ across the two developmental time points. Finally, by including the assessment of alpha-synuclein, a protein involved in the development of Lewy bodies, and a metabolite of lipid peroxidation (i.e. F₂-isoprostanes), we sought to provide insight into whether or not changes in brain iron

biology brought on by high fat diet-induced obesity set the stage for future neurological impairment. The overall study design is depicted in Figure 1.2, below.

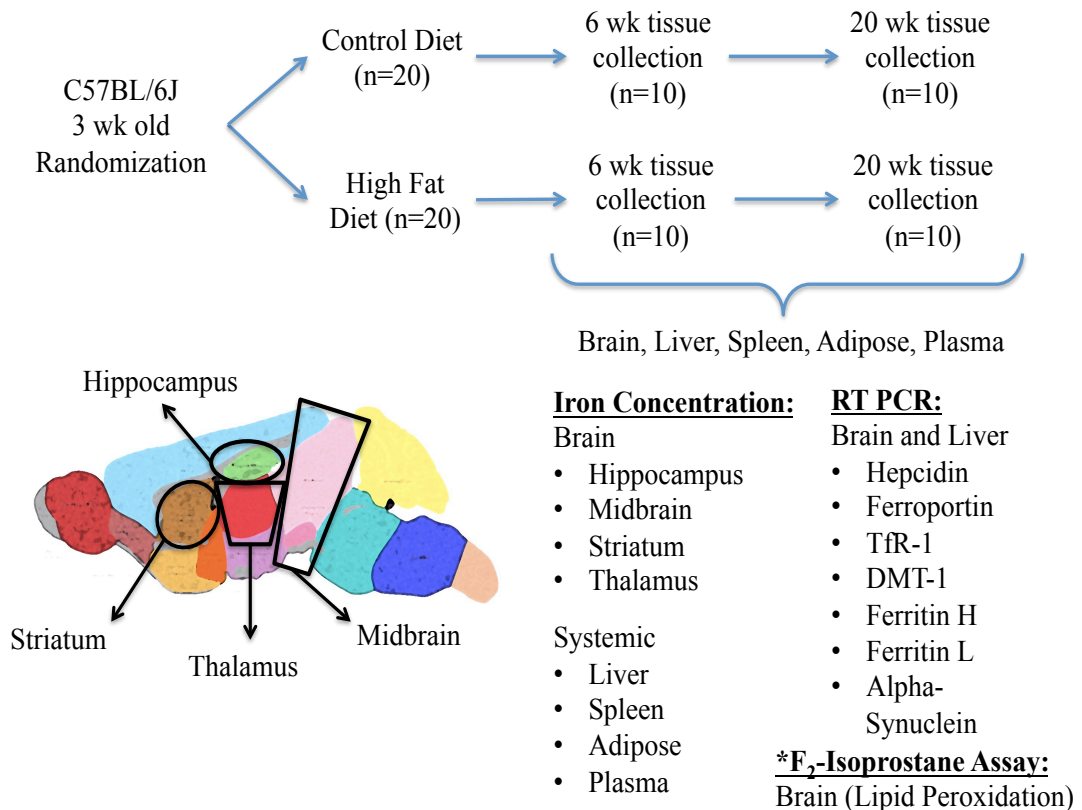


Figure 1.2. Study Design. Twenty-one day old male C57BL/6J mice (n=40) were randomly divided into two dietary groups, high fat diet (60% of Kcal from fat) and low fat control diet (10% Kcal from fat). After 6 weeks (6 wk) of feeding, brain, liver, spleen, and plasma samples were collected from ten adolescent mice in each dietary group. The tissues were assessed for iron concentration ($\mu\text{g}/\text{mg}$ protein) and iron-related gene expression. Sample collection and analysis was repeated after 20 weeks (20 wk) of diet in the remaining adult mice. Separately, lipid peroxidation metabolites were assessed in the 20 week midbrain and hippocampus using an F₂-isoprostane assay conducted by the lab of Dr. Michael Aschner. (Brain image courtesy of www.gensat.org)

CHAPTER II

REVIEW OF THE LITERATURE

Introduction

Iron is an essential dietary element that, when associated with select proteins, helps to safely transport oxygen, metabolize macronutrients, and synthesize proteins including neurotransmitters in the human body. Iron's ability to support these functions comes largely from its ability to participate in single electron chemistry by readily converting between its ferrous (Fe^{+2}) and ferric (Fe^{+3}) forms. This same attribute allows free, unbound iron to catalyze the generation of reactive oxygen species that can be detrimental to human health. The body has developed an array of proteins to govern the metabolism of iron; however, this system is susceptible to environmental influences that may result in poor iron management.

Obesity is known to increase the risk of systemic iron deficiency in a manner that is not fully understood [1,2]. Further, the effect of obesity on brain iron biology has yet to be reported. The following literature review will discuss what is known about the normal biology of iron in the brain, the effects of altered iron status on regional brain iron concentration, associations between obesity and systemic iron status, as well as associations between obesity and neurodegeneration. The cumulative evidence indicates that obesity has the potential to alter iron status in the brain, likely in a regional manner,

and that the susceptible brain regions are implicated in neurodegenerative diseases for which obesity is a risk factor.

Brain Iron Transport

The unique anatomy of the brain including the blood brain barrier (BBB), glial cells, ventricles, and cerebrospinal fluid produces an environment that is distinct from the remainder of the body. Despite these physical distinctions, the brain expresses many of the same iron metabolism proteins that are active elsewhere in the body including the transport protein transferrin (Tf), the storage protein ferritin, the export protein ferroportin, as well as the membrane receptor proteins transferrin receptor -1 (TfR-1), and divalent metal transporter-1 (DMT-1) [8,24,39,40]. Currently, the complete mechanism of iron transport throughout the brain is not fully understood. The following will provide an overview of key concepts relevant to the current study.

Iron predominantly enters the brain at the BBB, a system of microvasculature related to the capillary system found elsewhere in the body. Structurally, the BBB can be thought of as a multi-layer system which coordinates to form a unique and highly selective barrier transport system [reviewed in [41]]. Research indicates that the predominant pathway for iron to enter the brain parenchyma is through the action of TfR-1 on the luminal side of the capillary endothelial cells that form the BBB [42,43]. The path across the endothelial cell involves the formation of an endosome around the holotransferrin molecule where iron is released by decreased pH [44]. It is notable that the existence of DMT-1 in the BBB endothelial cells is debated [45–47]. Lack of DMT-1

would require participation of an undetermined transporter to release iron from the lysosome into the cytoplasm. Once free of the endosome, iron is available for transport out of the epithelial cell by ferroportin; however, it must be oxidized by either ceruloplasmin or hephaestin prior to entering the brain circulation [40,48]. Studies using radiolabeled iron show that the holo-transferrin is not shuttled through the endothelial cell [49]. Rather, the apo-transferrin TfR-1 complex is predominately transported back into the luminal side of the endothelial cells where apo-transferrin is released into the plasma[49].

The brain is fully equipped to distribute iron even without the influx of systemic iron transport proteins. Brain-derived transferrin is produced by neurons, oligodendrocytes, astrocytes, and the choroid plexus and thought to act as the primary intercellular transporter of iron throughout the brain [8,50,51]. Further, neurons, astrocytes and microglia express DMT-1 and TfR-1, allowing for the accumulation of transferrin bound iron as well as non-transferrin bound iron [27,39,52]. Neurons, astrocytes, oligodendrocytes, and microglia all express ferroportin, which functions as the primary transporter of iron from the cells into the brain circulation [40,48]. The ventricles and choroid plexus have also been shown to be involved in brain iron homeostasis. Studies demonstrating the transport of iron into tissues proximal to these structures indicate that they may play a role in brain iron accumulation and export via the cerebrospinal fluid [8,53].

In summary, iron biology in the brain shares similarities with systemic iron biology including transport proteins, cell surface receptors and the regulatory protein

hepcidin. It is therefore possible that environmental stimuli, which have an effect on systemic iron biology, may also result in changes to brain iron biology. However, studies of iron deficiency and repletion indicate that the regulation of iron in the brain is dynamic and involves the consideration of both developmental stage and brain region.

The Brain and Iron Status

Research indicates that brain iron biology varies throughout development and that brain iron accumulation is associated with the normal aging process [7–10,54]. The effects of insufficient iron during the early stages of brain development and cognition have been studied and are known to cause impairments that can last decades despite iron supplementation [11,55]. Conversely, the pathology of neurodegenerative diseases including Alzheimer's disease and Parkinson's disease are characterized by accumulations of iron in brain regions associated with resultant cognitive or motor control impairment. Specifically, Alzheimer's disease is associated with iron accumulations in the hippocampus and Parkinson's disease with accumulations in the substantia nigra, part of the midbrain [28,29,33–35,38]. While these findings underscore the importance of maintaining appropriate iron levels in the brain, much remains unknown about how the brain responds to altered iron status and the long-term impacts on brain health.

Regional changes in brain iron during deficiency and repletion has been studied in Sprague-Dawley rats [9,10]. These studies demonstrated the effects of altered dietary iron during early developmental time points (birth to 9 weeks). Interestingly, the regional

effects of iron deficiency varied by study with a significant decrease in iron concentration and increase in Tf expression in the hippocampus alone in the first study [9]; versus significant iron concentration decreases in the substantia nigra, striatum and thalamus in the later study [10]. It is notable that the thalamus only responded to deficiency at later time points. Response to deficiency and repletion was also heterogeneous within regions in the second study with the hippocampus responding to supplementation, but not deficiency; while the substantia nigra and striatum generally responded to deficiency but not supplementation. Each of these studies used intakes of iron deficient diets that yielded severe iron deficiency in a vulnerable developmental stage. The effect of chronic, low-grade iron deficiency on regional brain biology is unknown and may differ from the observations above, particularly at later stages of life when development slows. While there still is more to be learned about regional brain iron biology during development, the concept of a differentiated response to environmental stress may support a role for the iron alterations brought on by obesity in the development of brain region specific neurodegeneration.

Obesity and Iron Status

Currently, the Center for Disease Control estimates that over a third of adults and 17 percent of children in the United States are obese and at increased risk for co-morbid disease [56]. Indeed, evidence indicates that obesity increases the risk of adult onset diabetes, cardiovascular disease and cancer (reviewed [57]) as well as neurological disorders [13,15,16]. In the 1960s, two studies reported markers of decreased iron status

in obese adolescent boys and girls [58,59]. This finding was further explored in a 2004 analysis of the Third National Health and Nutritional Examination Survey (NHANES) [1]. The study found that the risk of iron deficiency increased with BMI and that children above the 85th percentile had approximately twice the risk of iron deficiency as those with normal BMI. This finding has been supported by several additional studies in diverse populations [6,60–63]. The NHANES results, published by Nead et al., are based on total iron deficiency, a measure that combined iron deficiency with anemia (IDA) and iron deficiency without anemia (ID) [1]. The group also reported the prevalence of IDA versus ID in each weight group, which showed that the majority of the affected obese individuals were not anemic (i.e. weight above 95th percentile, IDA = 1.1% vs. ID = 4.4%).

Obesity-associated iron deficiency has also been demonstrated in adults [4,22,23]. Studies of obese individuals undergoing gastric bypass surgery have reported rates of preoperative iron deficiency as high as 35% to 43.9% [22,23]. These studies further indicate that, within obese populations, younger individuals and women have a higher prevalence of iron deficiency than men [22,23]. The higher rate of ID in obese women as compared to men is consistent with increased iron needs of women during reproductive years. However, the results of a small study (n=50) in postmenopausal obese women found elevated serum TfR-1, demonstrating that obesity-induced iron deficiency may persist in women beyond the reproductive years [64]. These results indicate that the prevalence of iron deficiency in the obese adult population is high and that younger individuals and women in general could be more at risk.

Attempts have been made to determine how obesity influences iron status. A systematic review of obesity's effect on iron status involving 25 studies concluded that obesity-related iron alterations exist and are suggestive of an inflammation-mediated pathway but too few studies included inflammatory markers to draw conclusions [2]. Findings in obese children (6 -14 years of age) in Switzerland found that changes in hepcidin levels and inflammation assessed by C-reactive protein (CRP) and interleukin-6 (IL-6) were driving the ID rather than low dietary iron intake [6]. Additionally, a convenience sample of 234 obese adults versus normal weight controls showed that the obese individuals had lower serum iron and higher serum TfR-1, CRP, and ferritin, findings consistent with inflammation-mediated iron deficiency [4]. While the mechanism has not been completely elucidated, the current understanding is that the chronic inflammation associated with obesity causes iron deficiency by increasing the iron regulatory proteins hepcidin and ferritin, which decrease iron absorption and recycling.

Animal models of obesity and altered iron status have focused on the causes of systemic iron deficiency including alterations in iron metabolism protein expression and poor absorption. Findings from a study using C57BL/6 mice fed low fat diet (LFD; 10% Kcal from fat) in the control group and high fat diet (HFD; 60% Kcal from fat) to induce obesity demonstrated decreased liver non-heme iron, increased expression of hepcidin mRNA relative to liver iron, and increased serum amyloid A, an inflammatory marker, in the high fat diet group [20]. A separate study using the same mouse model and diet showed high fat diet-induced obesity caused systemic ID independent of hepcidin [21].

The study found significant up-regulation of duodenal enterocyte DMT-1 and TfR-1 mRNA in the high fat diet groups without changes in hepcidin. In addition, two weeks of iron supplementation was sufficient to increase expression and serum levels of hepcidin in the low fat diet but not high fat diet mice. These findings indicate that the C57BL/6J mouse model of obesity develops markers of inflammation and iron deficiency.

Despite the lack of information on obesity and brain iron biology, studies looking at the effects of acute inflammation on brain iron biology provide support for a potential role. Recent studies have reported that the inflammatory cytokines tumor necrosis factor alpha (TNF- α) and IL-6, which are elevated in obesity, are capable of inducing the production of hepcidin in astrocytes and microglia as well as increasing DMT-1 in these cells and hippocampal neurons [6,19,24]. At the cellular level, the findings included significantly increased iron concentrations in the neurons and microglia. A separate cell study went on to show increased iron uptake and retention in astrocytes and microglia exposed to TNF- α [27]. Studies in rats further demonstrate that acute inflammation, unrelated to brain injury, alters brain iron biology. Both intravenous lipopolysaccharide and peripheral abscess have been shown to produce alterations in brain iron biology, regional variations in hepcidin expression, and increased expression of iron import and storage proteins within hours of administration [25,26].

While obesity is known to increase the risk of systemic iron deficiency, the effect of obesity on iron biology in the brain has not been studied [1,6,22]. Studies suggest that inflammation may play a role in the development of obesity-associated systemic iron deficiency [2,4,20]. Inflammation has also been shown to illicit changes in brain iron

biology in animal and cell culture studies [20,24–27]. However, it is unknown if obesity per se is sufficient to affect iron biology in the brain via inflammation or other physiological changes. Previous observations relating obesity, iron and neurodegenerative disease may provide support for further study as well as potential targets to assess in determining the relevance to human disease.

Obesity and Neurodegeneration

Emerging findings indicate a role for high fat diet and obesity in neurodegeneration with an etiology that is currently uncharacterized [13,65,66]. In a global analysis of the brain using magnetic resonance imaging, obesity was found to be negatively associated with gray matter and midbrain volume in a population of Japanese men ($n=690$, gray matter $P < 0.001$ and midbrain $P < 0.05$) [66]. Separately, evidence exists for the association between obesity and the two most prevalent neurodegenerative diseases: Alzheimer's disease and Parkinson's disease.

Alzheimer's Disease

Alzheimer's disease is the most common cause of dementia in individuals over the age of 65. Affecting over five million people in the U.S. alone, Alzheimer's disease had an estimated impact of greater than \$200 billion dollars in 2013 [67]. While the leading risk factors for Alzheimer's disease are advanced age (>60 years) and genetic predisposition, modifiable environmental factors including vascular disease, diabetes, and obesity have all been associated with an increased risk of Alzheimer's disease [13,68].

Further, a meta-analysis by Loeff and Walach summarized the findings of 13 studies to determine that midlife overweight and obesity significantly increased the relative risk for dementia by $RR = 1.34$ [95% CI 1.08, 1.66] and $RR = 1.91$ [95% CI 1.4, 2.62] respectively as compared to normal weight after adjusting for confounders [13]. In addition to being an independent risk factor for Alzheimer's disease, obesity is a known risk factor for many of the disease states associated with Alzheimer's disease and dementia including most prominently hypertension, and diabetes [57]. Associations between obesity and adult-onset diabetes further support the evidence for obesity's role in dementia. Meta-analysis and cohort studies have demonstrated that patients with an impaired glucose tolerance test, indicative of insulin resistance and diabetes, were at increased risk for Alzheimer's disease [69,70]. Despite these associations, the mechanisms driving the pathogenesis of Alzheimer's disease continue to be elusive.

Iron biology may offer some insight into the link between obesity and Alzheimer's disease. Beta-amyloid, a protein component of the plaques seen in Alzheimer's disease, has been shown to have ferroxidase activity [71]. Further, an iron response element has been documented in the 5' untranslated region of the gene for the amyloid precursor protein, the protein which is cleaved to form beta-amyloid [71,72]. Importantly, the presence of redox active iron in senile plaques and tangles has been demonstrated in Alzheimer's disease [73]. More recently, a series of studies demonstrated that gestational and neonatal systemic iron deficiency activates multiple Alzheimer's related genes in the hippocampus of mice and rats [74,75]. Further research is warranted to determine whether the alterations in brain iron biology brought on by

obesity are capable of influencing any of these targets, thus setting the stage for Alzheimer's disease.

Parkinson's Disease

Parkinson's disease is a chronic, degenerative movement disorder characterized by tremor, rigidity, slow movement, and postural instability that is the result of the loss of dopamine synthesizing cells in the substantia nigra, a brain region noted for high iron concentration [7,8]. Currently, the most prominent risk factor for Parkinson's disease is advanced age as only 5 - 10% of cases can be tied to known genetic mutations [76,77]. It is estimated that over one million people in the U.S. suffer from Parkinson's disease and that this number may continue to increase as the population ages [78]. Despite an increasing understanding of the pathophysiology of Parkinson's disease in recent years, the underlying etiology of the disease remains elusive, and there is no cure.

The role of obesity as a risk factor for Parkinson's disease is currently unclear. Prospective epidemiological studies of adiposity in midlife and resultant Parkinson's disease risk have had mixed results. In 2002, Abbot et al. found a significant association between midlife triceps skin fold thickness and future risk of Parkinson's disease in Japanese Americans using data from the Honolulu Heart Program [16]. Two years later Chen et al. was unable to find an overall association between BMI or central obesity and the development of Parkinson's disease using a data from the Health Professionals Follow-up Study and the Nurses' Health Study [15]. However, there were associations between measures of central adiposity and the risk of Parkinson's disease in the non-

smoking subset of this data [15]. Palacios et al. (2011) confirmed the lack of overall association between obesity, waist circumference, or diabetes and Parkinson’s disease using data from the Cancer Prevention Study II Nutrition Cohort, but notes that results may have been limited by biases of self-reporting [79].

Environmental exposure risks for Parkinson’s disease include increased relative risk factors for diets high in animal fat, but the evidence is not conclusive. A recent case-control study in Japan showed some evidence of decreased risk of Parkinson’s disease in a dietary pattern described as “healthy” but no associations with “Western” or “light meal” patterns [65]. Previous studies looking at nutrient categories found increased risk of Parkinson’s disease related to high fat diets as summarized in Table 2.1.

Table 2.1. High Fat Diet and the Risk of Parkinson’s Disease

| Dietary Exposure | Risk: Odds Ratio (CI) | Study |
|--|--|--------------|
| Animal fat | 5.3 (1.8, 15.5) n=110 | [80] |
| High Animal Fat (ice cream, soft and hard cheese, butter, eggs, milk, beef, pork, liver) | 3.42 (1.38, 8.51) n=156 (note P = 0.15) | [81] |
| Total Fat | 1.94 (1.05, 3.58) N=126 | |
| Cholesterol | 2.11 (1.14, 3.90) N=126 | [14] |
| Iron | 1.88 (1.05, 3.38) N=126 | |

Finally, a study which looked at the relationship between iron and high dietary intake of animal fats found a nine fold increased risk of Parkinson’s disease when transferrin saturation was low and fat intake was high as compared to those with low fat

intake (OR, 9.0; 95% CI, 2.7-29.9) [82]. Further research is needed to clarify these results and determine what role high fat diets play, if any, in the development of Parkinson's disease.

Despite a lack of strong epidemiological data relating diet and obesity to Parkinson's disease, it is clear that alterations in iron biology are relevant to the disease. Elevated iron concentration in the substantia nigra is a consistent characteristic of the disease pathology [33–35]. Further, gathering evidence demonstrates that alpha-synuclein, a protein that has been implicated in Parkinson's disease by genetics and pathological findings, is also associated with iron metabolism [83–88].

Alpha-Synuclein

Alpha-synuclein is one of the primary components of Lewy bodies and Lewy neurites, which are aberrant intracellular protein accumulations [85]. Alpha-synuclein pathology is characteristic of a number of neurodegenerative diseases including: Parkinson's disease, Lewy body dementia, and Alzheimer's disease [85,89]. Recently, alpha-synuclein has been shown to associate with the neuronal presynaptic membrane and function in dopamine metabolism [90,91]. Further, alpha-synuclein has been shown to be more abundant in excitatory rather than inhibitory neurons in the hippocampus [92].

Work in animal models and cell culture has linked alpha-synuclein to iron biology as well as shown a potential role for the protein in the generation of reactive oxygen species. A small study, using the C57BL/6J mouse model of obesity, demonstrated that alpha-synuclein is upregulated in obese mice along with other dopamine transport related

genes [93]. Recently, alpha-synuclein has been shown to possess ferrireductase activity [87]; and a structure resembling an iron response element has been characterized in the 5' un-translated region of the alpha-synuclein gene [88]. Further, the most prevalent form of alpha-synuclein found in Lewy bodies involves the posttranslational phosphorylation of serine 129 [86]. This modification results in increased binding affinity for divalent metals including iron [94]. Pertinent to neurodegeneration, alpha-synuclein, but not other synucleins, has been shown to generate hydroxyl radicals when incubated with iron (II) in vitro [95].

Combined, the observations of increased risk of neurodegeneration and iron-related attributes in amyloid precursor protein as well as alpha-synuclein provide intriguing links between obesity and iron regulation in neurodegenerative disease. While a complete understanding of the etiology of neurodegenerative disease remains unclear, evidence relating alpha-synuclein and thus Lewy body pathology to obesity and iron biology is building. Assessing the impact of obesity on the expression of alpha-synuclein may provide useful insights into sporadic neurodegenerative disease.

Conclusion

As described in the preceding sections, associations between obesity, iron, and the proteins associated with neurodegenerative disease indicate a potential interplay that warrants further study. Obesity is known to alter systemic iron biology in children and adults. It is likely that obesity will impact brain iron since similar proteins actively regulate iron in the brain as in the remainder of the body and severe iron deficiency

results in decreased iron concentration in the brain; however, this is not certain. Further, overnutrition induced obesity is also associated with increased risks for Alzheimer's disease and Parkinson's disease. Each of these diseases is known to present with high iron concentrations in the affected brain regions. Finally, key proteins in Alzheimer's disease and Parkinson's disease have been shown to be influenced by obesity, and associate with iron.

Direct assessment is necessary to better understand the associations between obesity and brain iron biology. Such an assessment should take into account the regional nature of iron biology in the brain and observe any changes in the common iron regulatory proteins. Assessing changes in expression of a neurodegenerative disease related protein that is also associated with iron, like alpha-synuclein, as well as assays for oxidative stress would provide a insight into the relevance of altered iron status to disease states.

CHAPTER III

THE EFFECTS OF DIET-INDUCED OBESITY ON REGIONAL BRAIN IRON BIOLOGY

Abstract

The importance of iron homeostasis is particularly apparent in the brain where deficiencies result in impaired cognition and accumulations are associated with neurodegenerative diseases. Obesity is associated with increased risks for altered iron status systemically as well as neurodegenerative disease, but the effects of obesity on iron biology in the brain have yet to be studied. The following study examined the effect of obesity on brain iron biology by focusing on changes in regional brain iron concentration and the expression of iron metabolism proteins at two time points in a mouse model of obesity. C57BL/6J mice were fed a high fat diet for 6 or 20 weeks post weaning to induce obesity. Samples were collected at each time point, analyzed, and compared to control animals fed a low fat diet. The results indicated that obesity was sufficient to attenuate age-related changes in iron concentration seen in control animals between 6 and 20 weeks in the midbrain and thalamus, but not the hippocampus or striatum. Further, markers of neurodegeneration (i.e., alpha-synuclein mRNA expression, F₂-isoprostanes) were increased in the midbrain at 20 weeks in high fat fed mice as compared to controls. These results support previous findings that brain iron biology responds to environmental

stress in a regionally-distinct manner and indicates that alterations in brain iron biology due to obesity may be relevant in neurodegenerative disease

Introduction

Obesity is a growing international concern, and it is known that obese individuals experience alterations in iron status at an increased rate when compared with the normal weight population [1–3]. An association between obesity and iron deficiency has been demonstrated in children and adults in developed and underdeveloped countries [1,3,2,22]. As early as the 1960s, two studies reported decreased iron status in obese adolescent boys and girls in the United States [58,59]. Subsequent studies across diverse populations of children have supported these findings including an analysis of the Third National Health and Nutritional Examination Survey (NHANES), which found that obesity doubled the risk of iron deficiency in children [1,6,60–63]. A retrospective review of obese adult patients undergoing gastric bypass found that as many as 43.9% of patients were iron deficient [22].

It is known that iron homeostasis is important to brain health, with deficiencies resulting in cognitive impairment and regional excesses found in neurodegenerative processes [11,28,29,35,36,38]. Studies have sought to characterize some of the physiological changes associated with obesity altered iron biology; however, the effects of obesity on brain iron biology have yet to be studied [19,21,96,97]. Due to the brain's relative isolation across the blood brain barrier, the effects of obesity on iron biology in the brain may differ from that of the rest of the body [47,49,98]. Thus, we sought to

characterize the changes in brain iron biology brought on by high fat diet-induced obesity.

Inside the brain, cells synthesize many of the same iron transport and regulatory proteins found throughout the body [12]. For example, transferrin is synthesized endogenously by oligodendrocytes [50]; whereas neurons and glial cells, including astrocytes and microglia, express both transferrin receptor-1 and divalent metal transporter-1 [27,39,52]. Once inside the cell, iron is bound to ferritin, used by cytosolic proteins, or taken up by the mitochondria for several uses including ATP synthesis. The primary cellular iron exporter in the body, ferroportin, is expressed at the blood brain barrier, as well as in glial cells and neurons [24,27,40]. Hepcidin, which regulates ferroportin by causing its degradation, is produced by glia cells in the brain [24,99,100]. Further, these brain derived proteins react to inflammatory stimuli and signaling with regional variations resulting in heterogeneous changes in iron biology which are only beginning to be understood [24–27].

Animal studies have shown that changes in iron concentration also vary by brain region in response to dietary iron depletion and repletion as well as inflammatory triggers, which may be important to our understanding of neurodegenerative diseases [9,10,25,101]. Alzheimer's and Parkinson's diseases display the pathological finding of regional brain iron accumulation; however it is not known if this accumulation is causative or secondary to the disease process [28,29,33–35,38]. Recently, the iron regulatory proteins hepcidin and ferroportin were found to be reduced in the brains of Alzheimer's patients [102]. Previously, proteins involved in Alzheimer's disease

(amyloid precursor protein) and Parkinson's disease (alpha-synuclein) were shown to be effected by iron status, possess messenger RNA iron response elements, and display ferroxidase/reductase activity [71,72,75,87,88,103]. Beyond these protein related associations, the reactive nature of iron itself may be relevant to neuronal degradation as free or improperly stored iron is capable of generating free radicals with the potential to cause lipid peroxidation and cell death [73,95].

The most prevalent forms of neurodegeneration:, Alzheimer's disease and Parkinson's disease, have also been linked to obesity and high fat diets, though the mechanism(s) remain unknown. Specifically, midlife obesity has been shown by meta-analysis to increase the risk of Alzheimer's disease (BMI >30: R = 1.98 [95% CI 1.24, 3.41]) when compared to normal weight populations [13]. Other obesity-related risk factors including a poor dietary fat intake profile (e.g., low in omega 3 fats and high in animal and saturated fats) and insulin resistance have also been linked to an increased risk of developing dementia or Alzheimer's disease [69,104]. Parkinson's disease has been associated with increased adiposity as assessed by triceps skinfold thickness at midlife; but these results were not confirmed in subsequent studies using larger, more diverse populations [15,16,79]. However, dietary habits including higher intake of animal fats have been associated with increased risk of Parkinson's disease with increased odds ratio's ranging from 1.94 (CI: 1.05, 3.58) to 5.3 (CI: 1.8, 15.5) [80,82]. Together these findings indicate that modifiable environmental factors including diet and adiposity may prove to be relevant predictors or preventative targets for curbing the incidence of neurodegeneration in our aging population.

Here, we sought to characterize the effects of high fat, diet-induced obesity on regional brain iron concentration and regulatory protein expression in C57BL/6J mice at 6 and 20 weeks post weaning. Developmental research indicates that mice transition from the developmental stage into adulthood between 3 and 6 months of age [105]. The first time point was chosen to capture the cumulative effects of high fat diet-induced obesity during a period known to be vulnerable to changes in iron status (adolescent, late development); while the latter time point was chosen to assess effects in the mature brain where iron status is thought to be less critical (adulthood) [9,10]. The adult time point also provides physiologic data that may be used to support or refute previous epidemiological studies implicating midlife obesity as a risk factor for neurodegeneration [13,15,16]. The comparison of the data across two time points allowed for the observation of changes in regional iron concentration as the animals aged.

This study provides novel evaluation of brain iron biology using a well characterized mouse model of obesity to assess alterations in iron biology in four regions of the mouse brain that have been associated with Alzheimer's disease (hippocampus, thalamus), Parkinson's disease (midbrain-including substantia nigra, striatum) [7,8,28–36,38]. Additionally, alpha-synuclein gene expression and lipid peroxidation were assessed to determine if altered iron status was associated with in vivo markers of neurodegeneration [95,106].

Methods

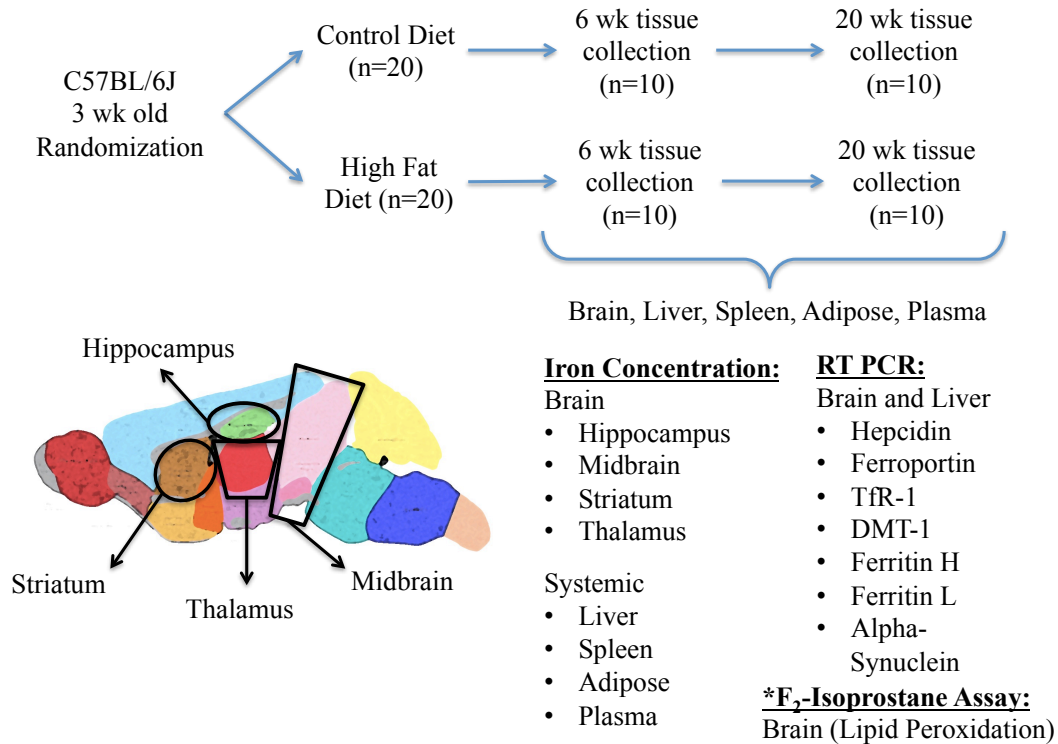


Figure 3.1. Study Design. Twenty-one day old male C57BL/6J mice (n=40) were acquired and randomly divided into two dietary groups, high fat diet (60% of Kcal from fat) and low fat control diet (10% Kcal from fat). After six weeks of feeding, brain liver, and spleen tissues as well as plasma samples were collected from ten mice in each dietary group. The tissues were assessed for iron concentration ($\mu\text{g}/\text{mg}$ protein) and iron metabolism related gene expression. Sample collection and analysis was repeated after twenty weeks of diet in the remaining mice. Separately, lipid peroxidation metabolites were assessed in the 20 week midbrain and hippocampus using an F₂-isoprostane assay conducted by the lab of Dr. Michael Aschner. (Brain image courtesy of www.gensat.org)

Animals

Prior to initiation of the study, approval for all animal care and procedures was obtained from the University of North Carolina at Greensboro's Animal Care and Use Committee. Male weanling (post-natal day 21) C57BL/6J mice (n=40) were purchased

from Jackson Laboratory (Bar Harbor, ME). The mice were randomly divided into two dietary groups, housed in pairs and provided bedding materials. Tail marking was used to identify 6 and 20 week groups from the beginning of the study. The housing environment was temperature controlled ($25 \pm 1^\circ \text{C}$) with automatic lights, which cycle off between 1800 h and 600 h. The mice were visually examined and weighed weekly throughout the study. The overall study design is presented in Figure 3.1 above.

Diet

Mice, housed by dietary group, were provided free access to diet and water 24 hours/day for 6 or 20 weeks. The control group was fed a low-fat diet (LFD; 10% kcal from fat, D12450B; Research Diets) and the experimental group received a high-fat diet (HFD; 60% kcal from fat, D12492; Research Diets) according to an established obesity model diet [17,18]. The source of the fat in the diets was lard. The diets included the same mineral mix (S10026; Research Diets), which provided 37mg of ferric citrate per 4057 Kcal. The similarity in iron content of the diets was confirmed by graphite furnace atomic absorption spectrometry (GFAAS) (Varian AA240, Varian, Inc., USA).

Hematocrit and Plasma

Blood from each mouse was collected in heparinized tubes at the time of sacrifice and stored on ice until processed. Hematocrit was determined by centrifugation of heparinized micro-hematocrit capillary tubes (Fisher Scientific; Waltham, MA).

Remaining whole blood samples were centrifuged for 15 minutes at 1000 x g to separate plasma for iron analysis. The plasma was stored at -80°C.

Tissue Collection

Adipose (epididymal, inguinal, mesenteric, and retroperitoneal depots), liver, and spleen were collected, weighed, and flash frozen at the time of sacrifice with help from members of the Dr. Michael McIntosh group.

The brain tissue from each mouse was dissected on an ice-cold tray at the time of sacrifice to isolate four iron-rich regions which are known to respond to alterations in iron status: midbrain, striatum, thalamus, and hippocampus [9]. Sample tissue was placed in pre-labeled, RNase free collection tubes. The liver, spleen and adipose were also collected, weighed, and placed in pre-labeled RNase free collection tubes. All tissues were flash frozen in liquid nitrogen and kept on dry ice for transport to a -80° C freezer where they were stored until processing.

Iron Analysis

Adipose, liver, spleen, and regional brain tissue as well as plasma samples were processed for iron analysis. The tissue samples were sonicated in cold radio-immunoprecipitation assay (RIPA) buffer with protease inhibitors to disrupt the tissue. Aliquots of the homogenate from each tissue sample were used to determine protein concentration using the Pierce Bicinchoninic Acid (BCA) Protein Assay (Thermo Fisher Scientific Inc., Rockford, IL) and separately digested in ultra-pure nitric acid (1:10 ratio)

for 48 hours in a sand bath (60° C). Aliquots (20 µl) of digested homogenate were further diluted in 2% nitric acid for analysis. Iron concentrations were measured using graphite furnace atomic absorption spectrometry (Varian AA240, Varian, Inc., USA). Bovine liver (NBS Standard Reference Material, USDC, Washington, DC) 184 µg Fe/g was digested in ultrapure nitric acid and used as an internal standard for analysis. All samples and controls were run in triplicate and outlier readings were excluded. Iron concentrations are expressed as µg Fe / mg protein.

RNA Isolation and cDNA Synthesis

RNA was isolated from the flash frozen regional brain tissue (right hemisphere) as well as weighed samples of liver tissue. The RNeasy® Lipid Tissue Mini Kit (Qiagen Inc., Valencia, Ca), QIAzol® Lysis Reagent (Qiagen Inc., Valencia, Ca), and stock chloroform were used to extract the RNA from all tissue samples according to the manufacturers protocol with the following alterations: tissues were sonicated in QIAzol Lysis Reagent; flow-through centrifugation was conducted for 30 rather than 15 seconds, where indicated; the optional membrane drying step was utilized; and 30 µl of RNase-free water was used to elute the RNA from the filter. Immediately following extraction RNA concentration and purity was determined by spectrophotometric analysis. An aliquot containing 2 µg of RNA was extracted from each sample and reverse transcribed using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, Ca) per the manufacturers protocol. Sample cDNA was used immediately or stored at -80° C.

Real-Time Polymerase Chain Reaction (RT-PCR)

RT-PCR gene expression assays were conducted with 20 ng of cDNA using TaqMan® Universal Master Mix II (Life Technologies, Carlsbad, Ca) and gene assays according to the manufacturer's protocol. The following iron metabolism related TaqMan® gene expression assays (Life Technologies, Carlsbad, Ca) assays were evaluated: hepcidin (Mm00519025_m1); transferrin receptor-1 (Mm00441941_m1); divalent metal transporter-1 (Mm00435363_m1); ferritin H (Mm00850707_g1); ferritin L (Mm03030144_g1); ferroportin (Mm00489837_m1); alpha-synuclein (Rn00560930_m1). All assays were confirmed as appropriate for use in murine species. Control and target assays were validated on excess sample tissue. 18s gene assay (Hs99999901_s1) was selected as the appropriate endogenous control. Relative gene expression was quantified using the delta-delta Ct method.

F₂-Isoprostane Assay

Isoprostane analysis was conducted in the lab of Dr. Michael Aschner at the Albert Einstein College of Medicine New York, NY using a gas chromatography/mass spectrometry with selective ion monitoring method previously described by Morrow and Roberts, 1999 and more recently outlined by da Silva Santos et al. 2014 [106,107].

Data Analysis

Body weight, iron concentration, and gene expression were analyzed using the statistical software package SPSS, Version 22 (Chicago, IL). Student's *t*-test was used to

evaluate mean differences in body weight and mRNA expression between LFD and HFD groups. A repeated measures ANOVA was used for the initial analysis of brain iron concentrations with distinct brain regions serving as the within group variable and diet as the between variable. This analysis was followed by a one-way analysis of variance (ANOVA), which was used to analyze the iron concentration data across time points and between diets within each brain region individually. The systemic iron concentrations were also analyzed using one-way ANOVA. The significance threshold was set at $P < 0.05$ for all analysis.

Results

Descriptive Data

Mice fed the HFD weighed significantly more than mice fed the LFD ($P < 0.001$) (Table 3.1). At 6 weeks HFD mice weighed 25% more on average than the LFD controls (HFD, 30.6 ± 0.4 g; LFD 26.2 ± 0.3 g; $P < 0.001$) and 32% more than LFD controls by 20 weeks (HFD, 52.2 ± 0.8 g; LFD, 39.6 ± 1.1 g; $P < 0.001$). An analysis of fat depots (combined: epididymal, inguinal, mesenteric, and retroperitoneal) showed a significant accumulation of white adipose tissue (WAT) in the HFD group as compared to LFD at both 6 and 20 weeks ($P < 0.001$) (Table 3.1). Liver weight was also significantly increased in the HFD group at 20 weeks compared to the LFD group (LFD 1.71 ± 0.14 g, HFD 2.80 ± 0.20 g; $P < 0.05$).

Table 3.1. Total Weight and Adipose Weight by Diet

| Age | Total Body Weight (g) | | Adipose Tissue (g) | |
|-------|-----------------------|--------------|--------------------|-------------|
| | LFD | HFD | LFD | HFD |
| 6 wk | 26.2 ± 0.30 | 30.7 ± 0.39‡ | 1.1 ± 0.11 | 3.0 ± 0.20‡ |
| 20 wk | 39.6 ± 1.12 | 52.2 ± 0.79‡ | 4.2 ± 0.32 | 7.1 ± 0.27‡ |

Body weight (6 wk, n=20; 20 wk, n=10) and combined adipose depot: epididymal, inguinal, mesenteric, and retroperitoneal weight (6 wk, n=10; 20 wk, n=10) in LFD versus HFD mice at 6 wk and 20 wk. Values are expressed as mean ± SEM. The “‡” indicates $P < 0.001$ versus LFD.

Systemic Iron Concentration

Iron concentrations for plasma and hematocrit were not significantly different between the groups (Table 3.2). Significant deficits in liver and spleen iron levels were present in the HFD group as compared to the LFD group at 6 and 20 weeks (liver: 6 wk and 20 wk, $P < 0.05$; spleen: 6 wk and 20 wk, $P < 0.001$) (Table 3.3.). Liver iron concentration was lower in the HFD group compared to the LFD group by 16% and 19% at 6 and 20 weeks respectively. Splenic iron concentration was lower by 45% at 6 weeks and 54% at 20 weeks as compared to LFD.

Table 3.2. Whole Blood Hematocrit and Plasma Iron Concentration

| Age | Plasma (µg/ml) | | Hematocrit | |
|-------|----------------|------------|-------------|-------------|
| | LFD | HFD | LFD | HFD |
| 6 wk | 5.4 ± 2.38 | 7.0 ± 1.41 | 0.47 ± 0.01 | 0.44 ± 0.02 |
| 20 wk | 5.1 ± 0.58 | 6.3 ± 0.59 | 0.48 ± 0.01 | 0.45 ± 0.01 |

Whole blood hematocrit (n=6) and plasma iron concentration (6 wk n=5; 20 wk n=6) in LFD versus HFD mice at 6 wk and 20 wk. Neither hematocrit or plasma iron concentration differed significantly between LFD and HFD groups at either time point. Values are expressed as mean ± SEM.

Table 3.3. Selected Systemic Tissue Iron Concentration (µg/mg protein)

| Age | Liver | | Spleen | | Adipose | |
|-------|-------------|--------------|-------------|--------------------------|-------------|-------------|
| | LFD | HFD | LFD | HFD | LFD | HFD |
| 6 wk | 0.51 ± 0.04 | 0.43 ± 0.01* | 2.09 ± 0.02 | 1.48 ± 0.08 [‡] | 0.56 ± 0.24 | 1.01 ± 0.24 |
| 20 wk | 0.65 ± 0.03 | 0.53 ± 0.03* | 4.38 ± 0.24 | 2.39 ± 0.16 [‡] | 0.64 ± 0.08 | 0.72 ± 0.16 |

Iron concentrations assessed by GFAAS in selected tissues from LFD versus HFD mice at 6 wk and 20 wk. Liver (6 wk LFD n=5, HFD n=6; 20 wk n=8) and spleen (6 wk LFD n=5, HFD n=6; 20 wk LFD n=9, HFD n=10) tissue iron concentrations were significantly lower in the HFD group at 6 wk and 20 wk. Adipose (6 wk n=5; 20 wk LFD n=5, HFD n=6) iron concentrations did not differ significantly. Values are expressed as mean ± SEM. The asterisk “*” indicates statistical significance at $P < 0.05$, and the “[‡]” $< P < 0.001$ versus LFD

Regional Brain Iron

A significant within-subjects main effect of brain region ($P < 0.05$) was observed using repeated measures ANOVA, indicating that the mean concentrations differed by region. Because there was a significant effect of region, further assessment of brain iron concentration was conducted for each brain region individually across time-points and between diets using one-way ANOVA. Analysis of the data for the individual regions indicated a significant effect of time in the midbrain and thalamus as well as significant overall difference between HFD and LFD in the midbrain ($P < 0.05$) (Figure 3.2.). A significant interaction of diet and time was observed the thalamus ($P < 0.05$). These findings indicate that fluctuations in iron concentrations from 6 to 20 weeks were attenuated in the HFD group as compared to the LFD group in the midbrain and thalamic regions. Hippocampus and striatum iron concentrations were unchanged by diet or time.

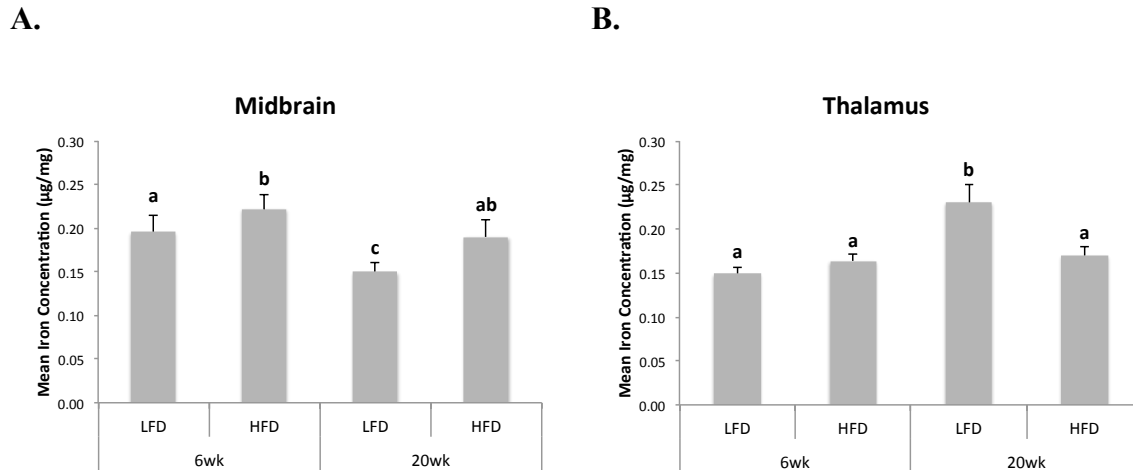
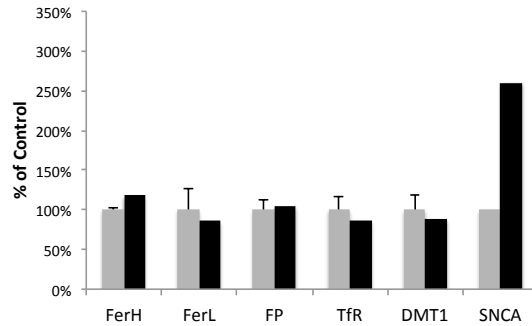


Figure 3.2. Select Regional Iron Concentrations. Iron concentrations in two brain regions where significant changes occurred with HFD treatment. A. Midbrain (6 wk, LFD n=9; HFD n=9; 20 wk, LFD n=8, HFD n=10) iron concentration was decreased in the LFD (control) between 6 and 20 weeks. This change in concentration was attenuated in the HFD group. B. Thalamus (6 wk, LFD n=9; HFD n=8; 20 wk, LFD n=9, HFD n=10) increased in the LFD (control) group from 6 to 20 weeks, a change that was again attenuated in the HFD group. Data were analyzed using one-way ANOVA and Student's *t* test. Means \pm SEM without a common letter differ, $P < 0.05$.

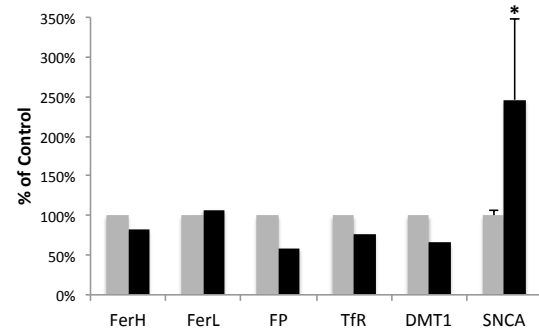
mRNA Expression of Iron Homeostasis Proteins

A comparison of iron transport and storage protein mRNA expression in HFD versus age matched LFD mice demonstrated few changes. Overall, there was some dysregulation at the 6 week time point in the HFD group. No significant changes in mRNA expression were observed in the striatum or the hippocampus at 6 or 20 weeks. Midbrain alpha-synuclein mRNA expression was elevated at both 6 and 20 weeks in the HFD group (Figure 3.3. A. & B.). The thalamus mRNA expression showed a significant elevation of alpha-synuclein in the HFD versus LFD groups at 6 weeks ($P < 0.05$) (Figure 3.3. C.), which was not present at 20 weeks (Figure 3.3. D.).

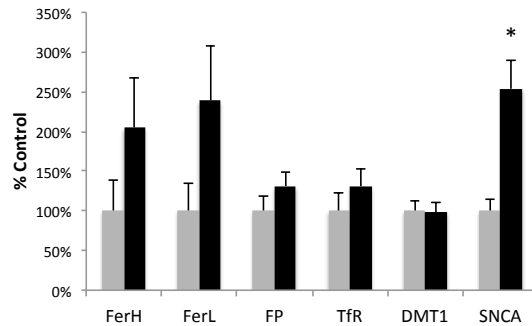
A. Midbrain 6 Weeks



B. Midbrain 20 Weeks



C. Thalamus 6 Weeks



D. Thalamus 20 Weeks

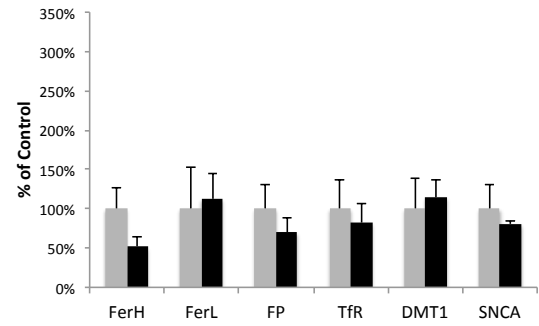
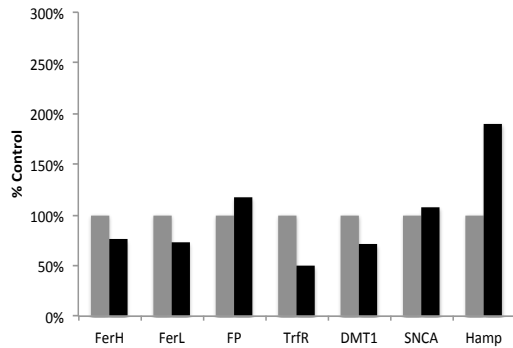


Figure 3.3. Brain Iron Metabolism Related mRNA Expression. LFD (gray) and HFD (black) Relative mRNA expression was quantified by RT-PCR using the delta-delta Ct method for ferritin H (FerH), ferritin L (FerL), ferroportin, transferrin receptor-1 (TfR), divalent metal transporter-1 (DMT-1), alpha-synuclein (SNCA). Regions assayed included A. midbrain 6 wk (LFD, n=3; HFD n=2; SNCA n=2) B. midbrain 20 wk (LFD, n=2; HFD, n=2; SNCA, n=4), C. thalamus 6 wk (n=3), D. thalamus 20 wk (n=3). Alpha-synuclein was elevated at 6 wk and significantly elevated at 20 wk in the midbrain as well as significantly elevated at 6 wk in the thalamus. Values are expressed as percent control \pm SEM. The asterisk “*” indicates statistical significance at $P < 0.05$ via independent samples *t*-test between LFD (control) and HFD groups.

Liver mRNA showed a decrease in transferrin receptor -1 and an increase in hepcidin expression of the HFD mice at 6 weeks. By 20 weeks transferrin receptor was similar between groups and hepcidin was decreased in the HFD compared to controls (Figure 3.4).

A. Liver 6 Weeks



B. Liver 20 Weeks

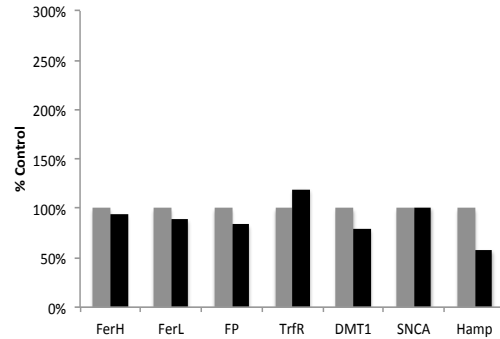


Figure 3.4. Liver Iron Metabolism Related mRNA Expression. LFD (gray) and HFD (black) Relative mRNA expression was quantified by RT-PCR using the delta-delta Ct method for ferritin H (FerH), ferritin L (FerL), ferroportin, transferrin receptor-1 (TrfR), divalent metal transporter-1 (DMT-1), alpha-synuclein (SNCA), and hepcidin (Hamp). Assays of the liver at A. 6 weeks (n=2) and B. 20 weeks (n=2) showed early, 6 week, elevation of hepcidin and decreased TrfR in the HFD group. By 20 weeks the expression TrfR was similar to LFD while Hamp was decreased in the HFD group. Values are expressed as percent control \pm SEM, with control based on expression in the LFD group.

F₂-Isoprostane Formation

Lipid peroxidation, as measured by F₂-isoprostane formation, was significantly elevated in the midbrains of the HFD exposed animals at 20 weeks as compared to LFD ($P < 0.05$) (Figure 3.5.). F₂-isoprostanes are stable metabolites of lipid peroxidation and thus serve as an indirect measurement of accumulated cellular membrane damage.

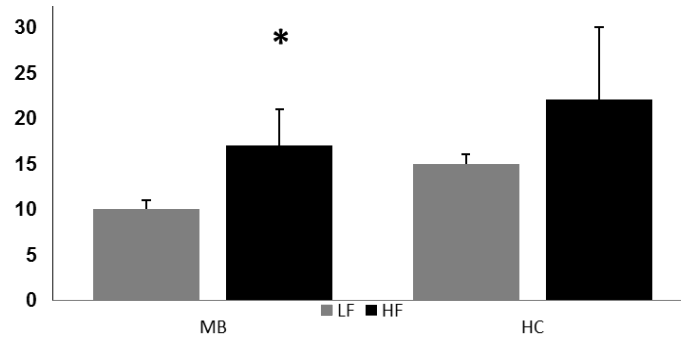


Figure 3.5. F₂-Isoprostane Assessment. F₂-Isoprostane concentration (ng/mg protein) provides an indirect measurement of lipid peroxidation products in the midbrain (MB) and hippocampus (HC) showed significant elevation in the midbrain at 20 weeks (n = 5). Values represent means \pm SEM; the asterisk “*” indicates statistical significance at $P < 0.05$ via independent samples *t*-test between LFD (gray) and HFD (black) groups.

Discussion

While the effects of obesity on systemic iron have been studied in animal and human models, our observations relating obesity to changes in brain iron biology are novel. Further, the systemic iron data that we gathered adds to, and corroborates the results of previous studies in the C57BL/6J mice showing that obesity alone is sufficient to affect iron status [20,21]. We found that the C57BL/6J model develops obesity-induced iron deficiency but not anemia, which is in agreement with the most common physiological presentation in the epidemiological data [1,22,23]. Further, the observation of altered regional iron concentration in conjunction with changes in alpha-synuclein expression and increased lipid peroxidation may implicate HFD induced obesity in neurodegenerative disease (Figure 3.6).

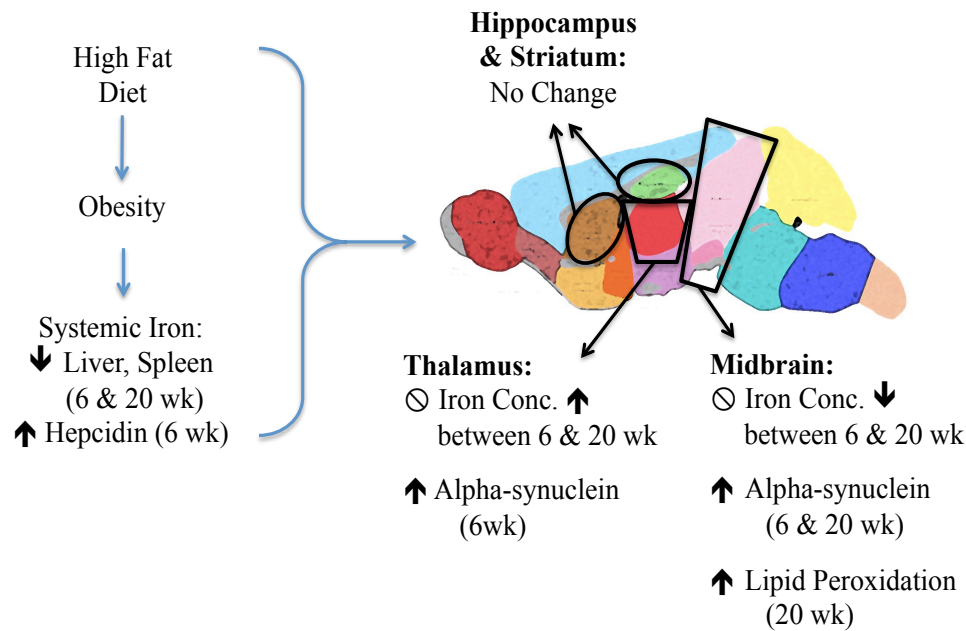


Figure 3.6. Results Summary. Key observations of this study include the following: 1) Mice fed a HFD had significant increased body weight and fat depot weight ($P < 0.001$) as well as altered iron status including decreased tissue iron concentrations in the liver ($P < 0.05$), and spleen ($P < 0.001$), with increased hepcidin expression in the liver at 6 weeks). 2) Iron biology was altered in the HFD midbrain and thalamus (attenuation of concentration change seen in the LFD, $P < 0.05$); 3) alpha-synuclein was elevated in the thalamus (6 wk, $P < 0.05$) and midbrain; 4) lipid peroxidation as assessed by F_2 -isoprostane assay was elevated in the midbrain ($P < 0.05$) of the HFD group compared to LFD. These observations indicate that obesity is sufficient to cause alterations in systemic iron biology with regionally differentiated results in the brain. Further, the brain regions where iron biology was altered also showed signs of neurodegenerative processes. (Brain image courtesy of www.gensat.org)

The proposed mechanisms for obesity-related alterations in iron biology include increased iron demand during growth, decreased dietary iron absorption, and inflammation-associated changes in iron regulation [3,6,21,24,26,108]. While each of these factors may play a role, much of the existing human research indicates that low-grade chronic inflammation alters iron biology [2]. Specifically, work by Yanoff et al. demonstrated that obese adults had lower serum iron, higher serum transferrin receptor,

and elevated C-reactive protein, which are associated with acute phase changes in iron biology [4]. Studies of dietary iron intake show that obesity alters iron status even when iron intake is sufficient, which argues against the dietary deficiency hypothesis and casts doubt on the notion that growth rate is the cause of the iron deficient status in obese individuals [3,6,21].

Mechanistically, the chronic inflammation associated with obesity is known to generate cytokines that increase serum hepcidin concentrations and trigger cellular iron sequestration. In particular, the cytokines TNF-alpha and interleukin-6 have been shown to increase hepcidin gene expression systemically and in the brain [24,26,109]. Previous studies in the C57BL/6J mouse model have reported increases in the same inflammatory markers in obese mice as compared to controls [19]. Our observations revealed increased liver hepcidin gene expression in the HFD group at 6 weeks as expected, but a decrease at 20 weeks relative to LFD. Previous studies in obese C57BL/6J mice have also found conflicting results for liver hepcidin gene expression ranging from no effect (8 weeks HFD) to decrease (16 weeks HFD) [20,21]. Chung et al. described a decreased expression of hepcidin at 16 weeks of HFD but went on to demonstrate that, relative to non-heme iron concentration in the liver, the hepcidin expression was actually higher in the HFD mice [20]. While unexpected, our 20 week finding was similar to the 16 week findings of Chung et al. where both hepcidin gene expression and liver iron concentration were decreased by HFD. These results indicate that liver tissue iron concentration may be important in regulating hepcidin levels during obesity.

Hepcidin is known to decrease cellular iron efflux by triggering the degradation of ferroportin, the only known cellular iron export protein [100]. Inhibiting ferroportin on the surface of intestinal enterocytes decreases the bioavailability of dietary iron, placing additional demands on endogenous iron to maintain homeostasis. In our study, low iron concentrations were found in the liver and spleen of HFD mice compared to LFD mice, corroborating previous findings [20]. Decreased iron concentrations in these tissues are indicative of systemic iron deficiency. However, the lack of a significant change in plasma iron concentration or hematocrit values indicates that anemia was not present despite tissue iron deficiency.

With the confirmation that the liver and spleen experienced alterations in iron concentration due to HFD induced obesity, we sought to determine if the iron-rich regions of the brain experienced similar changes. Iron is known to be distributed in a heterogeneous fashion across brain regions and is differentially affected by deficiency as well as repletion [7–10]. Further, induced inflammation in the rat results in a hepcidin response that differs by brain region with increases in the substantia nigra (midbrain) and cortex but not in the hippocampus or striatum [25]. However, these previous studies were each designed to evaluate acute or severe environmental stress: dietary iron deficiency or induced inflammation. Obesity is a chronic condition, which may produce different physiological changes in brain iron biology.

Using the C57BL/6J mouse model of obesity, we were able to demonstrate that HFD induced obesity altered iron concentrations in select regions of the brain when compared to LFD control. Our results support previous findings that brain iron biology is

region specific [9,10]. Repeated measures ANOVA confirmed that a significant difference in iron concentration was present in the four regions of the brain ($P < 0.001$) as well as an interaction effect of region and time ($P < 0.05$). Further analysis showed that HFD induced obesity primarily affected iron concentrations in the midbrain and thalamus with little impact on the hippocampus or striatum. These findings were comparable to previous work in weanling rats that showed varying the timing of an iron deficient diet from nursing, to early and late weaning affected which brain regions became deficient [10]. Specifically, the previous study results indicated that the hippocampus and striatum were vulnerable during the early pre-weaning period but not the post weaning period and the opposite was true for the thalamus. The same study also found that substantia nigra (part of the midbrain) was responsive to iron deficiency through the pre and post-weaning stages of development. Alternately, a similar study of iron deficiency in rats found that the hippocampus was the most affected region in the post-weaning period [9]. Our study differed from previous works because the variable of interest was diet-induced obesity and that each diet contained normal levels of iron (37 mg/kg) [110]. Despite the iron sufficiency of the diet, a significant change in iron concentration was found in the thalamus and the midbrain at the 20 week time point in the HFD mice compared to the LFD control mice. Interestingly, changes in the expression of alpha-synuclein mRNA fluctuated with the changes in iron concentration in the midbrain and thalamus while remaining unchanged in the striatum and hippocampus. This relationship will be discussed further in the following sections.

Obesity and Midbrain Iron

Taking a closer look at the effect of a HFD on the midbrain, our observations include four key alterations: an overall effect of diet on iron concentration; an attenuation of regional iron concentration changes resulting in an increase at 20 weeks; elevation of alpha-synuclein gene expression; and elevated F₂-isoprostanes. The elevated iron concentration in the midbrain of the HFD mice compared to the LFD mice is, to the best of our knowledge a unique finding. As discussed above, dietary induced iron deficiency anemia results in iron deficiencies in the midbrain [10]. However, in the unique setting of HFD induced obesity we observed an attenuation of the developmental decrease in midbrain iron seen in the LFD group. This attenuation resulted in a relative increase in midbrain iron as compared to the LFD group. This is in contrast to the expected result based on prior studies of dietary iron deficiency [9,10].

The results of our comparative RT-PCR and F₂-isoprostanes assays provided evidence that alpha-synuclein gene expression and lipid peroxidation were elevated in the HFD midbrains. Taken together, these observations are consistent with the pathological findings in Parkinson's disease where iron is known to accumulate in the midbrain (specifically the substantia nigra), alpha-synuclein aggregates to form Lewy bodies, and oxidative stress is implicated [33–35,85,95,111–113]. Based on these observations further assessment of the role of obesity in the etiology of Parkinson's disease is warranted.

Obesity and Thalamus Iron

Altered iron concentration and increased alpha-synuclein expression in the thalamus of HFD mice were also observed in our study. Iron concentration in the thalamus of the LFD mice increased between 6 and 20 weeks while in the HFD group it remained constant. The elevation of iron concentrations in the LFD control thalamus and the decrease in the LFD control midbrain further supports the dynamic regional regulation of iron in the brain during normal development. Gene expression of alpha-synuclein in the thalamus of HFD mice displayed a significant increase at 6 weeks which was not present at 20 weeks compared to the LFD group. Due to the relationship of alpha-synuclein to dopamine transport, our findings may have implications on thalamic dopamine biology as discussed below.

Alpha-Synuclein and Brain Iron

We evaluated the expression of alpha-synuclein, a protein associated with normal and cytotoxic dopamine biology as well as the formation of the Lewy bodies seen in Parkinson's disease due to the regional iron concentration changes in the midbrain and thalamus. Studies have demonstrated that alpha-synuclein has characteristics related to iron metabolism including ferrireductase activity and an iron response element [87,88,103]. Our observation that alpha-synuclein expression was affected in the brain regions that experienced changes in iron concentration is also indicative of a relationship between alpha-synuclein and brain iron. Alpha-synuclein is known to decrease with iron depletion, which may explain the drop in alpha-synuclein expression in the 20 week HFD

thalamus [103]. If the inverse is true, then increased expression of alpha-synuclein may confirm the relatively higher iron concentration in the midbrain of the HFD mice as compared to the LFD. Further, increased alpha-synuclein expression may also help to explain our finding that markers of lipid peroxidation were increased in the midbrain. As a ferrireductase, alpha-synuclein catalyzes the transition of iron to the active ferrous form increasing the risk of generating reactive oxygen species [87].

Alpha-synuclein is also known to affect neuronal dopamine transport in a manner related to obesity as well as neurodegeneration [93,113–115]. Increased expression of alpha-synuclein and dopamine biology related gene expression was previously reported in a model of obesity similar to our study [93]. Further, alpha-synuclein has been implicated the coordination of selective dopaminergic neurotoxicity [113]. Iron availability is also known to be important to dopamine biology, thus alterations in iron biology due to obesity may provide a means to study dopamine related neurotoxicity [37,116].

Implications and Future Study

In this initial assessment, we observed that chronic HFD-induced obesity had a regional effect on brain iron concentration as well as on markers of neurodegeneration in C57Bl/6J mice despite adequate iron availability in the diet. Our results show that HFD induced obesity has the ability to alter brain iron biology in a way that may be relevant to our understanding of neurological disease, particularly Parkinson's Disease. The key findings of attenuation of iron concentration changes (particularly in the midbrain but

also thalamus), increased alpha-synuclein expression, and increased lipid peroxidation in the midbrain fit well with the pathology of Parkinson's disease. The fact that these findings occurred at relatively early ages, adolescence (elevated alpha-synuclein) and adulthood (alterations in iron biology, elevated alpha-synuclein, and lipid peroxidation), suggest that mild chronic neurodegeneration may occur, possibly before clinical symptoms manifest. It is also notable that the obesity-related changes iron concentration occurred without iron deficiency anemia indicating that changes in the brain may be occurring in the absence of overt systemic manifestations. In this scenario the foundation of neurodegenerative disease may be developing for years prior to development of clinical symptoms.

Having determined that HFD induced obesity is sufficient to alter brain iron biology and markers of neurodegeneration, a broader approach is now necessary in order to determine the mechanisms involved and better assess the implications. Specifically, measurement of elevated regional glutathione activity, a key endogenous antioxidant, would further implicate iron-related reactive oxygen species generation as a cause of lipid peroxidation and subsequent neuronal loss in iron-rich areas. Histology of affected regions would provide evidence of physiological changes as well as identify the effects on specific cell types. Further assessment of proteins known to have links to iron metabolism and disease pathology including alpha-synuclein, amyloid precursor protein, and beta-amyloid protein would be valuable in understanding the etiology of neurodegeneration. Finally, iron deficiency and alterations in alpha-synuclein have been shown to impair dopamine transport. Thus, expanding the scope of these findings to

include effects on dopamine biology may provide a link between iron metabolic dysregulation and active disease stat

CHAPTER IV

EPILOGUE

The Erikson lab is focused on understanding how the altered biology of divalent elements including iron, copper, and manganese affect brain health. Previous unpublished data from our lab indicated that obese C57BL/6J mice had significantly higher concentrations of iron in the midbrain than control mice. The midbrain is known to be an iron-rich region that further accumulates iron in the pathology of Parkinson's disease [7,8,33–35]. Our lab sought to confirm and clarify the unpublished results in order to determine if obesity was sufficient to alter brain iron biology in a manner that might potentiate disease. During the study design, the scope was expanded from the preliminary findings to include four iron-rich brain regions known to be associated with neurodegenerative disease states: hippocampus, midbrain, striatum, and the thalamus in hopes of elucidating changes in other potentially affected areas [7,8,28–36,38]. The primary goals of the study were to: 1) assess the effects of HFD induced obesity on regional brain iron biology; 2) evaluate changes in regional brain iron at 6 weeks (adolescence) and 20 weeks (adult) time points, and 3) assess markers of neurodegeneration for potential links to disease states.

The theoretical model of our study was built on the knowledge that obese individuals experience systemic iron alterations and neurodegenerative disease at an increased rate [1–3,22]. Epidemiological data indicates that the alteration in iron

biology is likely due to the effect of obesity-induced inflammation on the iron regulatory protein hepcidin [2,6]. Further, both iron deficiency and induced inflammation are known to effect iron biology in the brain in a regional manner; however, the effects of obesity per se on brain iron biology have not yet been reported [9,10,24,25]. Regional brain iron concentration is also known to be heterogeneously sensitive to altered iron status during development, particularly in regions that accumulate iron in cases of neurodegenerative disease [9,10,12,33–35]. Separately, studies have related obesity and high fat diets to increased risk of Alzheimer's disease and Parkinson's disease respectively, thus indicating that adiposity and diet have the potential to influence neurodegeneration, though the mechanism is unknown [13,80–82]. We sought to address gaps in the literature by determining what effect chronic HFD induced obesity has on regional brain iron and if changes in the brain might set the stage for neurodegenerative disease.

We hypothesized that alterations in brain iron biology brought on by HFD induced obesity may be relevant in neurodegeneration. To assess the effects of HFD induced obesity on brain iron biology we used a well-characterized animal model of obesity (C57BL/6J mouse) fed either a control LFD diet (10% Kcals from fat) or an experimental HFD (60% Kcals from fat) iron sufficient diet [17,18]. Care of the mice was shared between Steve Fordahl and Wan Shen (to whom we owe many thanks). The HFD intervention was started at weaning and the first samples were taken 6 weeks later, near the end of adolescence to assess changes during the developmental period. At this point the mice were already significantly heavier and showing signs of systemic iron deficiency (decreased iron concentration in the liver and spleen, increased hepcidin expression in the

liver). While we did not find significant changes in brain iron concentration in these samples, we did note increased expression of alpha-synuclein in the midbrain and thalamus relative to the LFD controls. Alpha-synuclein, a protein with known associations to iron and dopamine biology, is a key constituent in Lewy bodies which are characteristic intracellular occlusions in Parkinson's pathology [72,86,87,90].

The second set of samples was timed to assess brain iron concentration in adult mice after prolonged exposure to the intervention. Interestingly, in these samples we found an attenuation of iron concentration changes in the midbrain and thalamus of the HFD group as compared to the LFD controls. Specifically, in the LFD controls the midbrain iron concentration decreased and the thalamus iron concentration increased while concentrations in the HFD intervention group remained unchanged from 6 to 20 weeks. Also, alpha-synuclein expression remained elevated in the midbrain but not the thalamus at 20 weeks. The 20 week results confirmed our previous findings that HFD induced obesity resulted in increased iron concentrations in the midbrain, but the finding that this was due to attenuation of iron efflux rather than accumulation is novel. Similarly, our finding that HFD attenuated iron concentration accumulation in the thalamus as compared to LFD is also new to the literature on brain iron biology.

Iron regulatory protein gene expression remained largely unchanged in the brain at either time point, while liver hepcidin expression showed signs of iron deficiency at 6 weeks. Our first attempt to extract RNA using a general kit resulted in low RNA concentrations that were unsuitable for PCR and thus several samples from the midbrain, thalamus, and hippocampus were excluded from analysis. A second method using a lipid

tissue specific kit was successful and resulted in sufficient RNA extraction for PCR. The lack of significant change in Ferritin H, Ferritin L, ferroportin, transferrin receptor-1, and divalent metal transporter-1 expression in the brain, despite iron concentration changes suggested that they were either not present or our study was underpowered to detect the changes. Additional methods of assessment (e.g., histology or western blot protein analysis) of iron-related protein distribution and concentration could help to clarify the mechanisms by which obesity effects iron status in the brain.

Overall, our observations demonstrate that HFD induced obesity is sufficient to cause systemic iron changes as well as alter iron biology in the midbrain and thalamus of C57BL/6J mice at 20 weeks post weaning. Further, the results confirm the regional nature of brain iron biology, as the midbrain and thalamus were affected while the hippocampus and striatum were not. The attenuation of iron concentration changes in the midbrain and thalamus due to HFD is new and needs to be confirmed. Additional study into the mechanism(s) linking HFD induced obesity to brain iron biology also should be pursued. We were also able to demonstrate that HFD induced obesity causes elevation of alpha-synuclein gene expression and that changes in alpha-synuclein were apparent in regions where iron concentration was also altered. The relevance of this finding is unknown, but we hypothesize that it may provide insight into the link between obesity and increased risk of neurodegenerative disease. Further work is needed to determine if these observations made during late adolescence and adulthood have relevance to disease progression typically associated with old age.

Additional research is needed to clarify the links between dietary induced obesity and iron metabolic profiles in the brain. Based on the information and results presented here the effects of developmental stage, duration of exposure, and brain region appear to be distinct and important. Determining if the alterations noted at 6 and 20 weeks relate to the duration of the diet or the developmental stage would provide valuable insight. Further, it would be useful to understand how changing the amount of iron or fat in the LFD and HFD affects iron biology systemically as well as in the brain.

Iron is not the only divalent metal important to normal brain function and an evaluation of diet-induced obesity on manganese and copper biology in the brain may provide additional insights. A study by Erikson et al. (2004) reported that dietary iron deficiency resulted in accumulation of divalent metals in the globus pallidus, indicating that alterations in iron could be a trigger for aberrant accumulations of other metals that affect brain health [101].

Recently proposed models of Lewy body development suggest a prion-like disease pathology where misfolded alpha-synuclein proteins both aggregate into cytoplasmic lesions as well as migrate and potentiate subsequent misfolding [117]. The model relies on a yet undefined precipitating event to trigger the cascade of Lewy body formation. A review of the effects of overnutrition by Cai et al. (2012) poses a link between overnutrition, hypothalamic inflammation, subsequent obesity and ultimately potentiation of neurodegeneration [118]. Based on the results presented here, iron biology may prove to be a link between such disparate hypotheses.

In conclusion these findings support previous work indicating that obesity alters iron biology systemically and advances our understanding of the effect of obesity on brain iron biology as well as neurodegeneration. The results also support the epidemiological association between obesity and altered iron status using an animal model. Further, this work provides evidence of links between obesity, iron biology, and markers of neurodegeneration.

Future study may make it possible to hone in on the mechanisms driving these alterations and thus fundamentally contribute to the understating of brain iron biology in states of health and disease. Since iron accumulation is readily identified in vivo using existing imaging techniques, the continuation of this work has the potential to develop diagnostic characteristics that may help to identify neurodegeneration prior to symptom onset and assess the effectiveness of treatment. Finally, this work may inform the discussion of the implications of overweight and obesity on long-term mental health

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